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Introduction

This project endeavors to create cell culture systems for the sensitive and rapid detection of prions. We have proposed to achieve this task by improving steps and components involved (i) in the infection of cells with prions, (ii) in the amplification of prions by cells, and (iii) in the detection of infected cells. To achieve these aims, we are trying to:

1. Identify or create improved cell lines that are more susceptible to prion infection.
2. Improve the delivery of prions to cells by improving their presentation and method of administration.
3. Increase the level of prion/PrP^{Sc} amplification in the infected cells.
4. Design better ways to detect prion infection in cells, either by increasing the formation of PrP^{Sc} or by devising new, non-PrP^{Sc} "surrogate" markers.

We now report on our efforts and results in these various approaches during the first year of the project. Our main achievements can be summarized as follows:

- Working with the persistently infected lines ScN2a and ScGT1 cells, we have found very promising ways to increase PrP^{Sc}, and in some cases also PrP^{C}, by more than 1 order of magnitude (for each treatment alone). In our hands, this translates in a vast improvement of signal-to-noise ratio in PrP^{Sc} detection both by immunofluorescence and in western blots. Whether this will also reflect in the susceptibility of cells to infection remains to be seen.
- We have experimented in novel ways to administer prions to cells using metallic support. These efforts are still in progress.
- We have obtained subclones of ScN2a cells with vastly improved levels of PrP^{Sc}. We are now examining whether curing these cells from prions may yield cell clones that are inherently more susceptible to prion reinfection. Variants of GT1 and of C6 cells are also under scrutiny.
- Looking for surrogate markers for prion infection, we have found that infecting GT1-1 cells leads to a vast *increase* in the neurosecretion SNARE proteins, synaptophysin and SNAP25. These findings are included in a manuscript sent for publication in the framework of the present project (attached) (Sandberg and Löw, submitted). We are now looking into ways to incorporate this finding into a prion detection system.

1. Increasing PrP^C and PrP^{Sc} in cells.

The purpose of this effort is to increase the production of PrP^{Sc} in order to improve the infectibility of cells, and/or to facilitate the identification of prion infection by methods based on PrP^{Sc} detection.

We have identified several treatments that increase PrP^{Sc} in persistently infected ScN2a and ScGT1 cells (summarized in Table 1). In some cases, they act by increasing the level of the substrate, PrP^C, whereas some of these treatments act specifically to increase PrP^{Sc}.

Drug/treatment	Cell type	PrP^C	PrP^{Sc}	Optimal treatment	Maximal treatment length (days)
Trichostatin A (TSA)	N2a, GT1, and others	x 10	x 10	1-10 μM	1 d – followed by apoptosis
4-phenylbutyrate (4PBA)	N2a	x 10	x 10	6 mM	> 1 d
Distamycin A (DSA)	N2a	>x 10	>x 10	20 μM	> 4 d
BDNF (brain-derived neurotrophic factor)	ScGT1	No change	x 2	100 ng/ml	> 4 d
Cysteine protease inhibitors	ScGT1	No change	x 10	1.5-15 μM	4d
Optimized culture media	ScGT1, ScN2a	In some cases glycoforms change	x 2-10		> 4 d

Table 1: Treatments that increase the formation of PrP^{Sc} in persistently infected cells

a. Trichostatin A (TSA). This histone deacetylase inhibitor has been shown to increase the expression of PrP^C through the activation of the PrP promoter. We have examined its action on the production of PrP^{Sc} in persistently infected cells. In both ScN2a and ScGT1 cells, TSA increased both PrP isoforms by at least 10 folds both in western blots (Fig. 1) and by immunofluorescence microscopy (Fig. 2). Remarkably, this increase held not only for endogenous PrP, which is transcribed under the control of its own promoter, but also for PrP placed under the CMV promoter in a standard expression vector (pCIneo). We are now examining this phenomenon.

As an illustration of the potential practical implications of the TSA treatment, we have used it to discover undetectable PrP^{Sc} in ScN2a cells that had been "cured" by the application of sterol analogs. A one day treatment with TSA produced a very strong PrP^{Sc} signal, which was absolutely invisible in untreated cells (Fig. 3, compare lanes 3 in upper (untreated) and lower (treated) cells). This demonstrated that the curing was incomplete. Indeed, when the sterol inhibitor was removed for several days, PrP^{Sc} became detectable again in the cells (Fig. 3, lane 3 and 4).

Toxicity: In ScN2a clones, treatment at the optimal doses appears to be limited to 1 d, after which cells undergo apoptosis. We have experimented with intermittent administration of the drug.

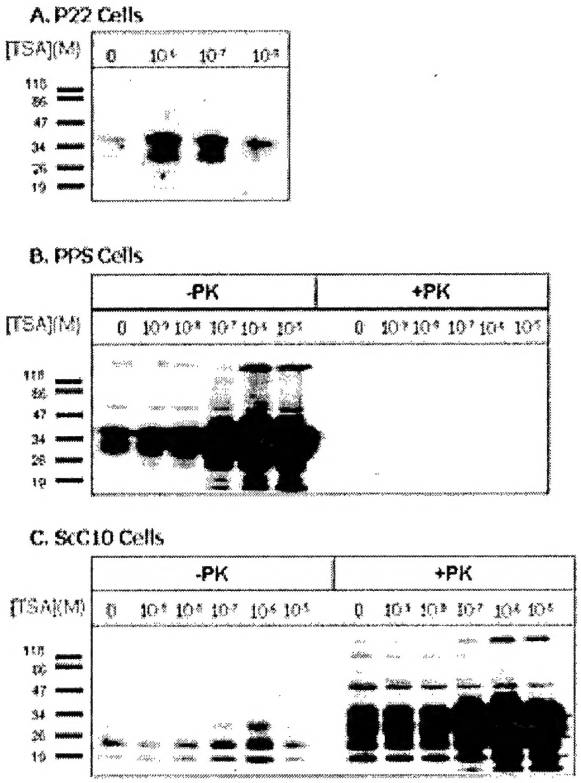


Figure 1: Vast increase in both PrP isoforms in N2a cells treated with trichostatin A (TSA). Cells were treated for 24 h with the indicated concentrations of TSA, and the PrP isoforms were then detected in western blots developed with the mAb 3F4. All cells overexpress the 3F4-labeled mouse PrP under the control of a CMV promoter. P22 are non-infected cells, whereas PPS are ScN2a cells that were cured from prions by a 1 week treatment with pentosan polysulfate. PK: proteinase K (20µg/ml, 1h, 37°C prior to electrophoresis).

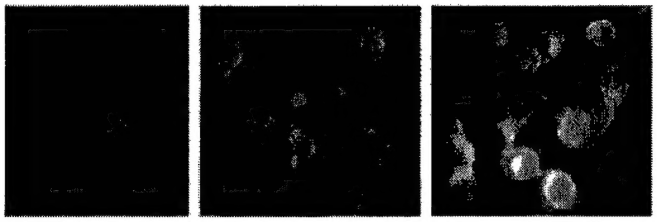


Figure 2: PrP^{Sc} increase after TSA treatment. ScN2a-c10 cells were treated for 1 d with TSA at the concentrations indicated. PrP^{Sc} was the detected by established guanidine immunofluorescence procedures.

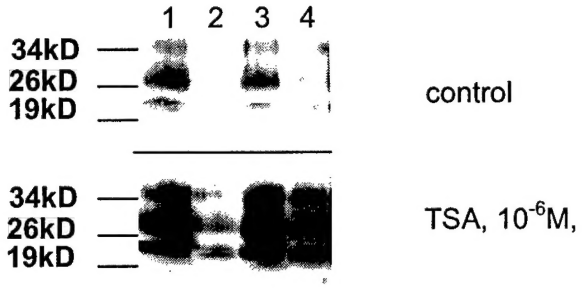


Figure 3. TSA reveals undetectable PrP^{Sc} in partially cured cells. ScN2a-c10 cells were treated with a sterol analog for 14, 7 or 10 d (lanes 2-4). In lanes 3 and 4, the sterol analog was removed and cells were left to recover for 7 or 4 d, respectively. In the bottom panel, cells were treated with TSA for 1 d prior to analysis. Protease-resistant PrP^{Sc} was then detected in a western blot developed with 3F4. In lane 2, undetectable PrP^{Sc} (upper panel) is clearly revealed by the TSA treatment. Lane 4 also illustrates the vast PrP^{Sc} increase caused by TSA.

b. Sodium 4-phenylbutyrate (4PBA) is a transcriptional regulator with pleiotropic effects. We were originally motivated to experiment with 4PBA by reports that it can relieve the trafficking block of mutant CFTR, perhaps by decreasing the levels of Hsc70 in treated cells (Rubenstein and Zeitlin, 2000). In our hands, 4PBA increased the levels of both PrP isoforms by at least 10 folds (Fig. 4). We are now studying the toxicity of this compound.

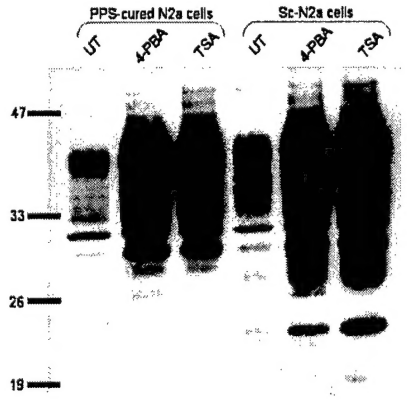


Figure 4: 4PBA increases PrP in both cured (PPS) and infected (ScN2a) cells. Cells were treated for 1 d with 6.25 mM of 4PBA. Samples were analyzed without prior proteolysis.

c. Distamycin A (DSA) is an antiviral minor groove binder that interacts with DNA in A-T rich regions (see e.g. (Baraldi et al., 2004)). While DSA often reduces transcription, we found unexpectedly that it increases both PrP isoforms by at least one order of magnitude (Fig. 5). We are now trying to determine its mode of action. Unlike TSA and 4PBA, distamycin A is **not lethal** in N2a cells. Rather, treated cells gradually increase in size and appear to stop dividing, but they remain viable for more than 5 days. We envisage that these features may be advantageous for the projected tissue culture test, where if would be helpful to have a large amount of PrP^{Sc} eventually concentrated in a single cell.

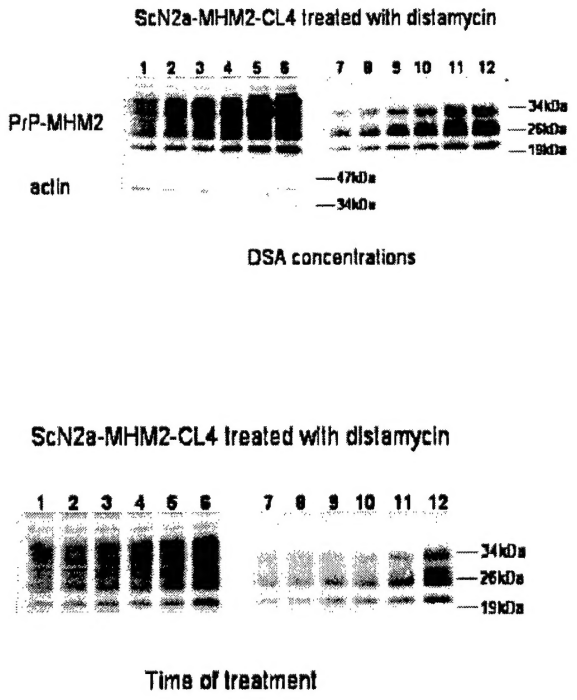


Figure 5: Distamycin A increases both PrP isoforms in a time- and concentration- dependent way. ScN2a-c10 cells were treated with DSA, and then total PrP (lanes 1-6) or protease-resistant PrP^{Sc} (lanes 7-12) were detected by western blotting (3F4). Upper panel: cells were treated for 4 d with 0, 1, 2, 5, 10, or 20 μM DSA (lanes 1-6 and 7-12). Lower panel: Cells were treated with 10 μM DSA for 0, 6, 14, 24, 48, or 96 h prior to analysis.

d. Brain-derived neurotrophic factor (BDNF). This factor is expressed predominantly in the nervous system. The levels of the factor can be increased by increased synaptic activity and at the same time BDNF can cause an increase in synaptic activity creating a vicious circle that has implication for the development of epilepsy. Since epileptic activities are a main clinical feature of Creutzfeldt-Jakob disease we analyzed the involvement of BDNF in prion infections. In the first series of experiments we found that scrapie infection of GT1-1 cells caused an increase in the levels of mRNA encoding BDNF in these cells. Interestingly, when ScGT1-1 cells were treated with BDNF (up to 100 ng/ml) for 4 days, there was a striking increase in PrP^{Sc} (Fig .6). Remarkably, there was no concomitant increase in PrP^{C}, suggesting that it is a prion-specific pathway that is altered by the growth factor. These observations indicate that a vicious circle could be induced during prion infections in neuronal cells, i.e. the infection enhances the production of BDNF which in turn enhances the formation of prions. We are now trying to determine the mode of action of BDNF on prion formation defining the intracellular signaling system involved.

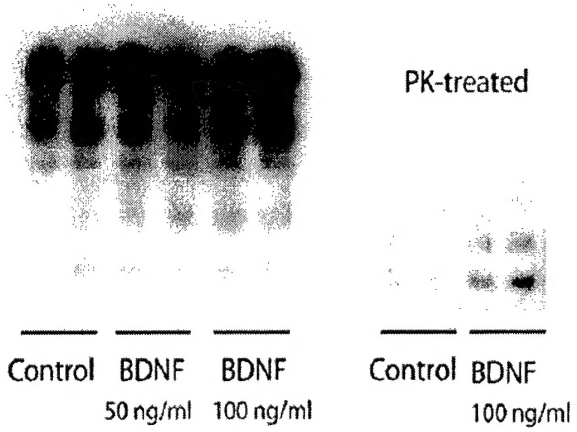


Figure 6: BDNF increases PrP^{Sc} in ScGT1-1 cells. Cells were treated with the indicated levels of BDNF for 4 days, and total (left) or proteinase K-resistant PrP were detected by western immunoblots developed with the recombinant Fab D18.

e. Cysteine protease inhibitors. We have recently reported that both exogenous and endogenous PrP^{Sc} are degraded in cells by cysteine proteases (Luhr et al., 2004b)including the lysosomal cathepsins B and L (Luhr et al., 2004a). In ScGT1-1 cells, treatment with leupeptin and E64 c and d caused increases in the level of PrP^{Sc} (Luhr et al 2004a). In particular, a combination of the inhibitors for cathepsin B and L, i.e. the cysteine protease inhibitors Z-Phe-Tyr-aldehyde (cathepsin L inhibitor) and L-trans-Epoxysuccinyl-Ile-Pro-OMe propylamide (CA074Me; cathepsin B inhibitor) cause a marked increase (>x10) in the levels of PrP^{Sc} (Luhr et al., 2004b). These treatments will be integrated in later development stages of the system.

f. Optimized culture media. We have found that altering the growth medium of persistently infected cells profoundly alters their levels of PrP^{Sc}. For instance, PrP^{Sc} levels in ScGT1-1 cells were vastly increased by increasing the serum concentration (Fig. 7A), and even more so by replacing the standard DMEM medium with the Neurobasal B27 formulation (panel B).

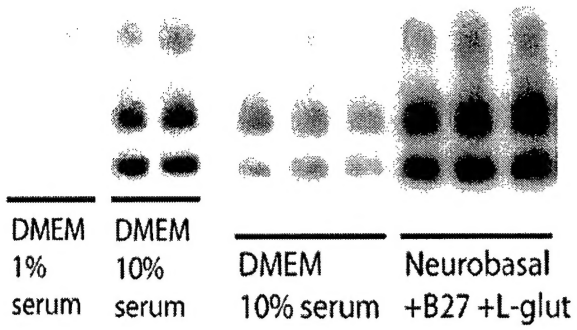


Figure 7. The cell growth medium determines PrP^{Sc} in ScGT1-1 cells. The cells were maintained in the indicated media for 72h prior to PrP analysis in western blots developed with the D13 recombinant Fab. Increasing serum concentration (A) and by switching to the Neurobasal B27 medium (B) both vastly increased protease-resistant PrP^{Sc}.

2. Surrogate markers

Hitherto, PrP^{Sc} has been the sole known marker of prion infection in cells. Single cell detection of PrP^{Sc} is achieved at the microscopic level using guanidine-enhanced immunofluorescence. However, we are operating under the assumption that prion infection might eventually cause much more observable effects in cells, thus amplifying the effect of prions. A plaque-like assay where cell death is the observable would of course be ideal, but we are trying to identify other cellular parameters that can be strictly correlated with prion infection.

In search for such surrogate markers we have focused on proteins involved in neurotransmitter vesicle release, because in the scrapie-infected brains an early involvement of synaptic vesicle releasing proteins has repeatedly described. In ScGT-1 cells we have now found that the levels of SNAP-25, synaptophysin and syntaxin 1A proteins were increased compared to uninfected GT1-1 cells. Increased mRNA levels of both splice variants SNAP-25a and b in ScGT1-1 cells were seen. There was no apparent difference in the morphology or neuritic outgrowth of the GT1-1 and ScGT1-1 cells. In addition, no difference in the distribution of SNAP-25, synaptophysin or syntaxin 1A was observed between ScGT1-1 and uninfected GT1-1 cells using immunohistochemistry, either in untreated cells or in cells further differentiated with dibutyryl cyclic AMP and 3-isobutyl-1-metylxanthine. Treatment with quinacrine or pentosan polysulphate cleared the abnormal isoform of the scrapie prion protein (PrP^{Sc}) from the ScGT1-1 cell cultures and also reversed the increased level of SNAP-25 and synaptophysin, indicating that PrP^{Sc} causes the altered protein expression. These results indicate that a scrapie infection can cause changes in the expression of proteins involved in neuronal secretion (manuscript attached) (Sandberg and Löw, submitted).

3. Enhancing the infection of cells with scrapie.

The initial step in infecting cells with prions is their exposure to prion-containing material. This step is notoriously inefficient and we are investing considerable efforts in trying to (i) decrease the degradation of the inoculum in cells and (ii) find improved ways to inoculate prions into cells.

We have recently shown that both endogenous and exogenous PrP^{Sc} are degraded in cells by cysteine proteases. We are thus planning to examine the feasibility to include relevant inhibitors (either chemical inhibitors or using a siRNA approach (Luhr et al., 2004a)) in the infection step. Although the inoculum is clearly stabilized by such treatments (Fig. 8), we do not know yet if this may results in the increased infection of cells.

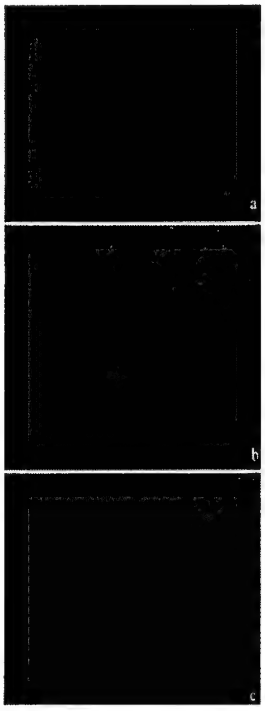
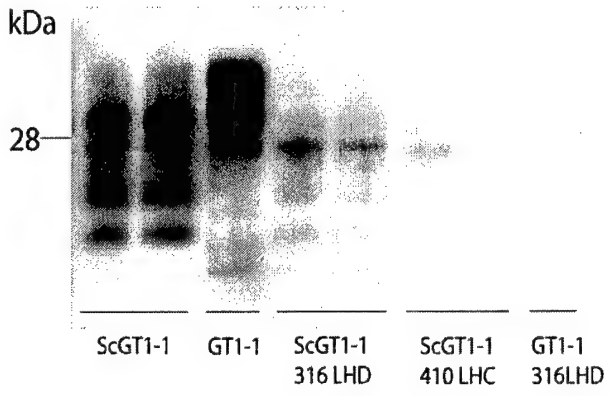


Figure 8: The cysteine protease inhibitor leupeptin slows the degradation of exogenous PrP^{Sc} in cells. Homogenate of ScGT1-1 cells with (c) or without (b) 15 μM leupeptin was added to GT1-1 cells growing in 35 mm dishes (a) and the cells were kept for 3 days and then fixed, treated with Triton X-100 and 3M GdnSCN and PrP were detected using Fab D13 with Cy3 goat anti human Fab.

Using metallic support to enhance prion infection.

A major goal of this project is to devise more efficient ways to present the infected material to cells. Current methods of cell infection are derived from virology procedures, were the material to be titrated is added to a minimal volume of medium and further incubated with the cells. This method relies on the very efficient interactions of the virus with its cellular receptors. However, because this technique is notoriously inefficient for prions, we are trying to devise ways to concentrate the infectious prions and bring them closer to the cells. In the first year of the project we have experimented with steel particles and with commercial magnetofection ™ reagents.

- **Absorption of PrP^{Sc} onto steel particles.**
  We were originally motivated in these experiments by the observation that prions attach tightly to surgical steel, and that such prions are highly infectious both in brains and in cultured cells (Flechsig et al., 2001; Zobeley et al., 1999). We reasoned that by providing a very large surface area, steel powder could perhaps multiply these effects. We have experimented with several types of steel powder of various compositions, and especially with two formulations: 316 LHD and 410 LHC (Höganäs AB, Höganäs, Sweden). These particles (Fig. 9, lower panel) are about 50 μ across, and we have confirmed that they are not phagocytosed by cells (e.g., Fig. 10). Both absorb PrP^{Sc} from scrapie-infected cell homogenates upon long (up to 36h) incubation (Fig. 9).



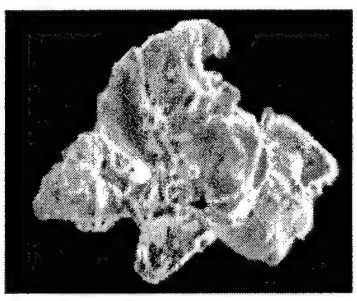


Figure 9: Absorption of PrP^{Sc} to steel particles. 50 μM homogenates of ScGT1-1 or GT1-1 cells were mixed with 5 ml of serum free medium and the steel powder. They were then shaken for 36 hrs at RT. The steel powder was then rinsed in PBS, boiled in 300 μM of loading buffer, and the supernatant was analyzed in western blots developed with the D13 Fab. Both powder types, but especially the 316 LHD, attach some PrP^{Sc} as revealed by the characteristic PrP^{Sc} electrophoretic pattern. Left 4 lanes: Cell homogenates included as controls. Bottom panel: a 316 LHD particle examines by SEM (from the producer's website: www.hoganas.com).

Attempts to infect cells with these particles have been disappointing so far (data not shown).

- **Magnetofection.**

Extensive experimentation with the magnetofection reagents POLYMAG and COMBIMAG from Chemicell (www.chemicell.com) have also failed to enhance the infection of cells, so far (data not shown).

4. Creation of cells lines with increased susceptibility to prions

One of the most frustrating conundrums of prion cell biology is that there is no known reason for the paucity of cell types that can be infected. With the discovery of novel accessory molecules of prion replication, new avenues of inquiry open up. In addition to PrP^C, which is an obvious prerequisite, accessory molecules and structures include (i) cholesterol rafts (Taraboulos et al., 1995), (ii) cellular proteoglycans (Ben-Zaken et al., 2003), and the laminin receptor (Leucht et al., 2003). We will start soon to use these parameters in a systematic study of cell lines and clones. In a preliminary study of rafts in susceptible cells, we noticed that cured ScN2a-c10 cells have much more floating PrP^C than the normal N2a population (Fig. 10). Whether there is a general correlation between susceptibility and the proportion of floating PrP^C remains to be seen.

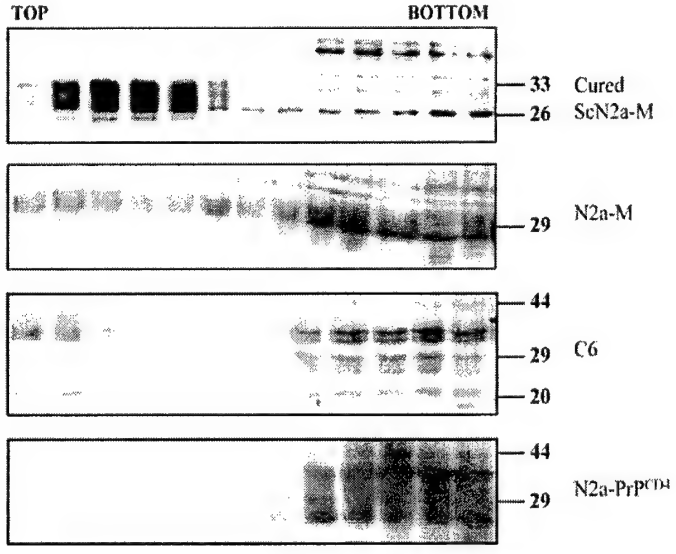


Figure 10: Vastly increased flotation of PrP^C in cured ScN2a cells. The indicated cells were lysed in 1% TX-100, subjected to a flotation assay in Nycodenz, and PrP in the fractions was detected by western immunoblotting. PrP^{CD4} was included as a non-raft marker. All PrP^C completely floated in cured ScN2a cells, as opposed to the other lines. (ScN2a were cured with pentosan sulfate)

- **PrP overexpression in existing cell lines**

Since PrP is an obvious prerequisite for susceptibility towards prions, we have constructed several cells lines that overexpress the 3F4-labeled mouse PrP. Clones with satisfying expression were obtained with GT1 and with C6 cells (Fig. 11).

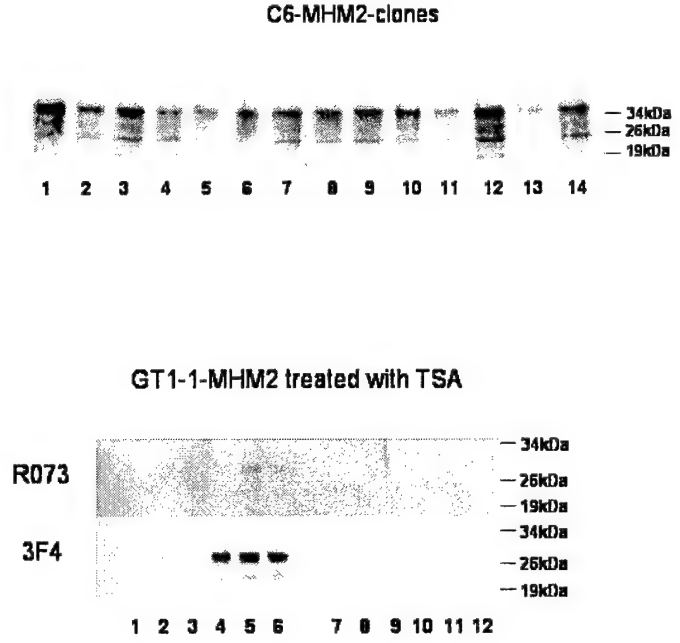


Figure 12: Construction of PrP overexpressing clones. The 3F4-labeled PrP-MHM2 was stably transfected into C6 rat glioma cells (upper panel) and into GT1-1 cells (lower panel). Western blots revealed a satisfying PrP expression in C6 cells (lanes represent single cell clones). PrP expression in GT1-MHM2 was considerably increased by a 1 d TSA treatment (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} µM, lanes 1-6 and 7-12, respectively). Compare lanes 4-6 to lanes 1-3), but no spontaneous protease-resistant PrP^{Sc} was formed in these clones (lanes 7-12).

- **Selection of better ScN2a clones**

We have embarked upon the isolation of subclones of ScN2a-c10 cells that produce increased amounts of PrP^{Sc}. Several such subclones have been stably producing PrP^{Sc} for several months (data not shown).

Conclusions

Our major success in the first year of this project has been in increasing PrP^{Sc} in infected cells. We have devised several (and unexpected) procedures that increase PrP^{Sc} by 1 order of magnitude when used individually. Whether these procedures can be used synergistically will be examined during the next few months. We have also discovered proteins that are vastly increased during prion infection of GT1-1 cells. We will now study the feasibility to use this variation as a surrogate marker for prion infection (for instance, by measuring secretion from the cells). These efforts will integrate into the original work plan.

In contrast, we have been less successful in our attempts to use metallic support for the efficient inoculation of prions to cells. We will now broaden our efforts on this topic

In the next year, we will start to integrate our results into a rudimentary system for the detection of prions.

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Altered Expression of Proteins involved in Neurosecretion in Scrapie-infected GT1-1 Cells

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Running Title: Neurosecretion regulatory Proteins and Scrapie infection

ABSTRACT

Prions cause transmissible and fatal diseases, which are associated with spongiform degeneration, astrogliosis and loss of axon terminals in the brains. To determine the expression of proteins involved in neurosecretion and synaptic functions after prion infection, gonadotropin-releasing hormone neuronal cell line subclone (GT1-1) was infected with the RML scrapie strain and analyzed by Western blotting, real time PCR and immunohistochemistry. The levels of SNAP-25, synaptophysin and syntaxin1A proteins were increased in scrapie-infected GT1-1 cells (ScGT1-1) compared to uninfected GT1-1 cells. Increased mRNA levels of both splice variants SNAP-25a and b in ScGT1-1 cells were seen. There was no apparent difference in the morphology or neuritic outgrowth of the GT1-1 and ScGT1-1 cells. In addition, no difference in the distribution of SNAP-25, synaptophysin or syntaxin 1A was observed between ScGT1-1 and uninfected GT1-1 cells using immunohistochemistry, either in in untreated cells or in cells further differentiated with Dibutyryl cyclic AMP and 3-isobutyl-1-metylxanthine. Treatment with quinacrine or pentosan polysulphate cleared the abnormal isoform of the scrapie prion protein (PrP^{Sc}) from the ScGT1-1 cell cultures and also reversed the increased level of SNAP-25 and synaptophysin, indicating that PrP^{Sc} causes the altered protein expression. These results indicate that a scrapie infection can cause changes in the expression of proteins involved in neuronal secretion, which may be implicated in the reduced N-type calcium channel responses described previously in scrapie-infected GT1-1 cells. In addition, these changes observed may be of pathogenic relevance for the axon terminal changes seen in prion-infected brains.

INTRODUCTION

Prion diseases are neurodegenerative diseases that can be transmissible, inherited or of sporadic occurrence. They are neuropathologically characterized by a marked astrogliosis, spongiform degeneration and neuronal loss in the brain, for review see (1). The vacuoles that give the spongiform character of the degeneration are mainly located to dendrites, which also show varicosities and loss of spines as revealed in Golgi impregnated specimens (2). In addition to these dendritic changes, reduced expression of presynaptic marker proteins, such as synaptophysin, synaptic-associated protein of molecular weight 25,000 (SNAP-25), syntaxin 1, synapsin 1, and α- and β-synuclein, has been reported in both clinical materials (3-6) and animal experimental models (7,8) indicating that also presynaptic axon terminals are affected. The mechanisms behind the presynaptic changes and their potential pathogenetic role in the disease are not known. In order to clarify this, a cell culture system would be advantageous.

The GT1-1 cell line is an immortalized mouse hypothalamic gonadotropin-releasing neuronal cell line (9) and represents one of the few cell lines that can be successfully infected by prions (10). These GT1-1 cells express not only key proteins involved in the regulation of secretory exocytosis such as synaptotagmin, synaptobrevin and SNAP-25, but also synaptophysin that is localized in the membrane of small synaptic vesicles (11). Using this cell system, we have recently reported an impaired function of voltage-gated N-type calcium channels in prion-infected cells (12). Several studies have shown that synaptic vesicle release proteins i.e. syntaxin 1A and SNAP-25, can interact with presynaptic calcium channels (13,14) and it has also been reported that these synaptic proteins can modulate the function of presynaptic channels, (15-18). In the present study we asked whether alterations in the expression of proteins involved in neurosecretion or synaptic vesicle release could be

observed in prion-infected GT1-1 cultures. We here report that an increased expression of SNAP-25, synaptophysin and syntaxin 1A could be detected by Western blotting in scrapie-infected GT1-1 cells (ScGT1-1). In addition, when ScGT1-1 cell cultures were treated with quinacrine and pentosan polysulphate, the abnormal isoform of the prion protein (PrP^{Sc}) was cleared and the difference in expression of SNAP-25 and synaptophysin between treated ScGT1-1 and GT1-1 cells was reduced.

MATERIAL AND METHOD

Cell culture, infection and procedures to induce differentiation

The neuronal cell line GT1-1 (kindly provided by Dr. P. Mellon, The Salk Institute, La Jolla, CA, USA) was grown in 25 cm^2 or 75 cm^2 culture flasks (Corning Inc., Corning, NY, USA). Briefly, the cells were cultivated in Dulbecco's MEM containing Glutamax-I, supplemented with 5% horse serum, 5% fetal calf serum and 1% penicillin/streptomycin (supplemented DMEM; all obtained from Gibco, Paisley, UK), and maintained at 37°C in a humidified 5% CO_2 atmosphere. The medium was changed every 2^{nd} –3^{rd} day, and the cells were dissociated with trypsin (Gibco) and sub-cultured every 5^{th} day. For infection with scrapie, the cells were dissociated and seeded into 24-well plates (Corning Inc.), $4x10^3$ cells/well, and grown in supplemented DMEM. After reaching a 70-90% confluence, the cells were incubated with a brain homogenate from mice infected with the RML (Rocky Mountain Laboratory) strain of scrapie (obtained from Prof. S.B. Prusiner, Department of Biochemistry, UCSF, San Francisco, CA, USA). The homogenate was diluted 1:10 in supplemented DMEM and added to the cells for 72 h at 32°C. The medium was then removed, the cells cultivated in supplemented DMEM at 37°C and sub-cultured 5 times before they were analyzed for the presence of PrP^{Sc} by Western blotting. Uninfected GT1-1 cells were cultivated simultaneously

under the same conditions as the ScGT1-1 cells. To induce differentiation, GT1-1 cells and ScGT1-1 cells were plated in poly-L-lysine hydrobromide-coated (0.1 mg/ml; Sigma Chemical Co., St. Louise, MO, USA) 35mm x 10mm culture dishes (Corning Inc.), $5x10^5$ cells/dish, and grown in supplemented DMEM at 37°C for 2 days before 1 mM Dibutyryl cyclic AMP (db-cAMP; Sigma) and 200 µM 3-isobutyl-1-metylxanthine (IBMX; Sigma) were added to the culture media for 3 days.

Quinacrine and pentosan polysulphate treatment

For clearence of PrP^{Sc}, ScGT1-1 cells were grown in 25 cm^2 culture flasks (Corning Inc.) in supplemented DMEM (Gibco), together with 0.5µM quinacrine (Sigma) in 37°C in a humidified 5% CO_2 atmosphere. Fresh culture media and quinacrine were added every second day and the cells were sub-cultured every 5^{th} day for 2-3 weeks. Pentosan polysulphate (5 µg/ml; Sigma) was also used to clear PrP^{Sc} from the cultures. Fresh culture media and pentosan polysulphate were added every 3rd day, and the cells were sub-cultured every 5^{th} day for 2 weeks. Untreated ScGT1-1 cells and uninfected GT1-1 cells were cultivated simultaneously under the same conditions as the quinacrine or pentosan polysulphate-treated cells.

Western blot analyses

For detection of the normal (PrP^{C}) and the abnormal isoform of the prion protein (PrP^{Sc}), syntaxin 1A, synaptophysin and SNAP-25 Western blot analyses were performed. GT1-1 cells and ScGT1-1 cells were plated in poly-L-lysine hydrobromide-coated (0.1 mg/ml; Sigma) 35mm x 10mm culture dishes (Corning Inc.), $5x10^5$ cells/dish, and grown in supplemented DMEM at 37°C for 5 days. The cells were lysed in 100 µl lysis buffer containing 0.5% deoxycholic acid sodium salt, 0.5% Triton X-100, 150 mM NaCl and 10 mM

Tris-HCl, (pH 8.0; all obtained from Sigma) and a protease inhibitor cocktail (Complete, Mini, EDTA-free from Roche Diagnostics GmbH, Mannheim, Germany). The lysates were centrifuged at 13,000 rpm for 1 min and the pellets discarded. Protein concentrations were determined by a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). For analysis of PrP^{Sc}, the supernatant was split in two aliquots. One aliquot was treated with proteinase K (PK; Boehringer Mannheim, Mannheim, Germany), 10 μg/ml per 1 mg protein, for 30 min at 37°C. The proteinase K treatment exceeded the effects of the protease inhibitor cocktail and degraded the proteinase K sensitive scrapie prion protein. The PK degradation was stopped by incubation with 5 mM phenylmethyl sulfonyl fluoride (Sigma). Thereafter, the PK-treated and the other untreated aliquots were boiled in 4x SDS sample buffer (1x; 62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.003% bromophenol blue, 1% β-merkaptoethanol). The samples were loaded on NuPAGE 12% Bis-Tris gels (Invitrogen, Groningen, The Netherlands) and electrophoresed in NuPAGE MOPS SDS running buffer (Sigma) according to manufacturer's instructions. The gels were blotted to an Immobilon-P^{SQ} PVDF sequencing membrane (Millipore Corporation, Bedford, MA, USA). Primary antibodies used were D13 raised against PrP^{C} (HuM-Fab antibody, 1:700; InPro Biotechnology, Inc., San Fransisco, CA, USA), anti syntaxin 1A (mouse, purified IgG; 1:5000; Synaptic Systems, Göttingen, Germany), anti synaptophysin 1 (rabbit serum, 1:1000; Synaptic Systems) and anti SNAP-25 (rabbit serum, 1: 5000) (19). The antibodies were diluted in 0.3 % (w/v) Tween-20/Phosphate Buffered Saline solution (PBS-T; Sigma) and 5 % (w/v) Bovine Serum Albumin (BSA; Sigma). Secondary antibodies used were horseradish peroxidase-labeled goat anti-human IgG $F(ab')_2$ (Pierce, Rockford, IL, USA), goat anti-mouse IgG (DAKO Danmark A/S, Glostrup, Denmark) and swine anti-rabbit (DAKO), all diluted 1:5000 in PBS-T and 5% (w/v) non-fat dry milk. The blots were developed using the ECL+ detection system and Hyperfilm ECL (Amersham Pharmacia Biotech UK Limited,

Buckinghamshire, UK). Optical density was determined using Gel Doc 2000 with the software Quantity one 4.2.2 (Bio-Rad).

RNA extraction, cDNA synthesis and real time PCR

Cells were plated in poly-L-lysine hydrobromide-coated (0.1 mg/ml; Sigma) 35 x 10 mm culture dishes (Corning Inc.), 5.0 x 10^5 cells/dish, and grown in supplemented DMEM at 37°C for 5 days before experiments were performed. From these cultures, total RNA was extracted, using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The amount and purity of the RNA was assessed by spectrophotometry (Ultrospec Plus, Pharmacia LKB Biotechnology, Uppsala, Sweden). 500 ng of total RNA was subsequently treated with 1 unit of amplification grade DNase I (Invitrogen, Groningen, The Netherlands) for 15 min at room temperature and inactivated by the addition of 2.5 mM EDTA followed by incubation at 65°C for 10 min. The DNase treated RNA was reverse transcribed in a 20 µl reaction containing the following reagents from Invitrogen: 150 ng of random hexamer primers, 1 x RT buffer, 10mM DTT, 500 µM each of dNTPs and 50 U MoMLV reverse transcriptase (Superscript II). cDNA synthesis was allowed to proceed for1 h at 42°C before inactivation at 70°C for 15 min. For quantification of the relative mRNA levels of the SNAP-25a and b isoforms, primers corresponding to SNAP-25 [AB003991] exon 2, F (5′ to 3′ AGG ACG CAG ACA TGC GTA ATG AAC TGG AGG) together with SNAP-25a [AB003991] exon 5a, R (5′ to 3′ TTG GTT GAT ATG GTT CAT GCC TTC TTC GAC ACG A) and SNAP-25b [AB003992]exon 5b, R (5′ to 3′ CTT ATT GAT TTG GTC CAT CCC TTC CTC AAT GCG) were used (20). mRNA encoding cyclophilin was amplified using the following primers, F (5′ to 3′ GCT TTT CGC CGC TTG CT and R (5′ to 3′ CTC GTC ATC GGC CGT GAT) [X52803] were designed in Primer express. One µl of cDNA template was amplified in triplicate 25µl reactions containing the following reagents;

Platinum® SYBR® Green qPCR Supermix UDG and 250nM of each primer (all from Invitrogen). An ABI Prism® 7000 sequence detection system (Applied Biosystems) with the following cycling conditions were used, 95°C for 15 s and 60°C for 1 min, for 45 cycles. Real-time PCR data were analyzed using the ABI Prism 7000 software (Applied Biosystems). Data analysis were done using the $2^{-\Delta\Delta C}{}_T$ method for relative quantification (21) and all samples were normalized to cyclophilin that was used as an endogenous control.

Immunohistochemistry

The cell cultures were fixed in 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany) for 10 min and thereafter quenched in 0.1 M NH_4Cl (Sigma) for 5 min. The cultures were pre-incubated with 0.5% Triton-X100, 5% BSA and PBS for 10 min in room temperature. The cultures were then incubated with the rabbit polyclonal antibodies against synaptophysin 1 (1:50), or SNAP-25 (1:1000), or the mouse monoclonal antibody against syntaxin 1A (1:1000) diluted in 0.2% Triton X-100, 2.5% (w/v) BSA and PBS in 4°C over night. After washing, the cells were incubated with Rhodamin Red™-X-conjugated AffiniPure Donkey Anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and Cy™2-conjugated AffiniPure Donkey anti-rabbit (Jackson ImmunoResearch Laboratories, Inc.), diluted 1:500 and 1:250 in 0.2% Triton X-100, 2.5 % (w/v) BSA and PBS, for 1 h at room temperature. The cultures were mounted in glycerol containing 2.5% 1,4-diazabicyclo (2,2,2)-octane (Sigma). Images of the immunolabeled neurons were obtained with a CCD camera (AxioCam, Carl Zeiss Vision GmbH, Hallbergmoos, Germany) connected to a fluorescence microscope (Microphot FX, Nikon Corp., Tokyo, Japan) using AxioVision software (Carl Zeiss Vision GmbH) and prepared for illustration with Adobe software (Adobe Systems Inc., San Jose, CA, USA).

Statistics

Optical density and real-time PCR data were analysed using Student's *t*-test together with Welch's correction. All optical density data were converted to logarithmic values to get Gaussian distribution. Differences were considered statistically significant if $P< 0.05$ and bars represent mean values. All statistical analyses were made using Graph Pad Prism.

RESULTS

Scrapie-infected GT1-1 cells (ScGT1-1) showed an increased expression of SNAP-25, synaptophysin and syntaxin 1A compared to uninfected GT1-1 cells. When two ScGT1-1 cell batches infected at different time points were analyzed for the occurrence of PrP^{Sc}, the first ScGT1-1 cell batch (Sc batch A) showed a higher level of PrP^{Sc} than the other (Sc batch B) (Fig. 1A). There was no apparent difference in the morphology of GT1-1 and ScGT1-1 cells (Sc batch A) (Fig. 1B). By Western blotting, SNAP-25, synaptophysin and syntaxin 1A were seen in the GT1-1 cells (Fig. 1C-H), confirming previous observations on the occurrence of these proteins in these cells (9,11,22). The Sc batch A of ScGT1-1 cells showed an increase in the expression of SNAP-25 (27%), synaptophysin (16%) and syntaxin 1A (18%), 60-85 days post-infection (p.i.), compared to control cells (Fig. 1C-H), while the increase in expression of SNAP-25 in Sc batch B, which contained lower levels of PrP^{Sc}, was more modest, 60-70 days p.i. (Fig 1C, D). The following experiments were all performed using the ScGT1-1 cells (Sc batch A) showing the higher level of PrP^{Sc}.

ScGT1-1 cells exhibited an increased level of both the SNAP-25a and b isoform mRNA compared to uninfected GT1-1 cells. To investigate wether the increased SNAP-25 expression detected in ScGT1-1 cells was reflected at the mRNA level, real time PCR was

used. This method also made it possible to distinguish between the two different SNAP-25 isoforms (SNAP-25a and b). The mRNAs were determined to be SNAP-25a and b confirming previous results where GT1-1 cells have been found to express mRNA for SNAP-25 (9). The expression of the genes encoding SNAP-25a and b in ScGT1-1 cells, 100 days p.i., were elevated by 77 and 78% respectively, compared to uninfected GT1-1 cells (Fig. 2).

Db-cAMP / IBMX treated GT1-1 and ScGT1-1 cells showed a comparable increase in SNAP-25 and synaptophysin expression and were morphologically similar.

Using immunohistochemistry, the SNAP-25 labeling was mainly localized to the plasma membrane of the soma in a diffuse and non-clustered way, both in GT1-1 cells and ScGT1-1 cells (Fig. 3A, B). Also, a few neurites could be seen diffusely immunolabeled for SNAP-25 in both GT1-1 and ScGT1-1 cells. Synaptophysin labeling occurred as small puncta localized throughout the soma of the GT1-1 and ScGT1-1 cells. The neurites observed were also immuno-labeled (Fig. 3C, D).

An increased expression of SNAP-25 and neuritic outgrowth have been observed upon exposure of PC-12 cells to db-cAMP (23). To investigate wether ScGT1-1 cells were different from GT1-1 cells in this respect, we compared the effects of combined treatment with db-cAMP and IBMX in these cell populations. When GT1-1 cells and ScGT1-1 cells were treated with db-cAMP/IBMX for 3 days, outgrowth of neurites (Fig. 3E, F) as well as an increased expression of SNAP-25 and synaptophysin (Fig. 3G, H), were seen to a similar extent in both cell populations. The cellular localization of SNAP-25 and synaptophysin in the treated GT1-1 or ScGT1-1 cells showed no marked changes compared to the corresponding untreated cells although the number and length of neurites had increased markedly (Fig. 3E, F). These results indicate that the increased SNAP-25 or synaptophysin expression does not reflect a primary change in neuritic outgrowth in the cell cultures. .

Quinacrine and pentosan polysulphate cleared PrP^{Sc} from the ScGT1-1 cell cultures and reversed the increase in SNAP-25 and synaptophysin expression. Quinacrine and pentosan polysulphate treatment of prion-infected cell cultures have previously been shown to clear PrP^{Sc} from the cultures (24-26). Treatment of ScGT1-1 cells with 0.5μM quinacrine for 2-3 weeks caused a clearance of PrP^{Sc} from the cultures as shown by Western blotting (Fig. 4A). Such quinacrine treatment caused no marked changes in the levels of SNAP-25, synaptophysin or syntaxin 1A in uninfected GT1-1 cells and reversed the increased SNAP-25 and synaptophysin protein levels in the ScGT1-1 cells (85 days p.i.; Fig. 4B). However, the increased expression of syntaxin 1A in ScGT1-1 cells remained after quinacrine treatment (Fig. 4B). ScGT1-1 cells treated with pentosan polysulphate for 2 weeks showed no presence of PrP^{Sc} (data not shown). Also, in these cultures, the changes in expression of SNAP-25 and synaptophysin had become less evident, 110 days p.i., while the increased syntaxin 1A expression remained (data not shown).

DISCUSSION

In the present study marked alterations in the expression of proteins involved in neurosecretion in scrapie-infected GT1-1 cells was found. The GT1-1 cell clone, which is derived from hypothalamic neurons immortalized by genetically targeted tumorigensis using the promotor region of the gonadotropin-releasing hormone (GnRH) gene, expresses endogenous GnRH mRNA as well as neuronal cell markers like neuron-specific enolase (NSE) (9) and neuron-specific tubulin (TUJ1) (12). These cells contain two types of secretory vesicles involved in regulated secretion, namely large dense core vesicles (LDCVs) and small synaptic-like microvesicles (SLMVs) (11). While the LDCVs are involved in the release of

GnRH, the SLMVs have been shown to release GABA upon depolarization with extracellular K^+ (11). Synaptophysin, which is associated with SLMVs, shows a punctate pattern in the GT1-1 cells when immuno-labeled. SNAP-25, which is involved in both types of regulated secretion, is associated with the plasma membrane (11). In the present study, we observed a distribution of both SNAP-25 and synaptophysin similar to that previously described. The pattern of distribution of these two proteins and syntaxin 1A was comparable in the uninfected GT1-1 and ScGT1-1 cells. However, the level of SNAP-25, synaptophysin and syntaxin 1A expression in ScGT1-1 cells was markedly increased.

The increased level of SNAP-25 and synaptophysin was most likely a result of the presence of PrP^{Sc} in the cells, since the alterations were, at least partly, reversible upon treatment with quinacrine and pentosan polysulphate, which both abolished the PrP^{Sc}. In addition, the scrapie-infected and uninfected GT1-1 cells showed similar morphology and growth rate.

The protein SNAP-25 exists as two isoforms (27). SNAP-25a has been implicated in neuritic outgrowth and fusion of vesicles delivering components to the plasma membrane throughout the neurite and in the growth cones (28,29). SNAP-25b on the other hand, has been implicated in neuronal synaptic vesicle release and neuropeptide secretion in neuroendocrine cells (28,30). However, it has recently been shown that SNAP-25a also can be involved in secretory vesicle release (31).

In the present study we observed an up-regulation of mRNA for both isoforms during the infection, but no differences in growth or extension of neurites between uninfected and infected cells could be detected. When GT1-1 and ScGT1-1 cells were treated with cAMP, both cell populations extended neurites and showed a similar cellular distribution of SNAP-25 and syntaxin 1A. In addition, a comparable increase in SNAP-25 and synaptophysin level was seen in db-cAMP treated GT1-1 and ScGT1-1 cells using Western blotting. Thus, there was

no evidence that the observed changes in SNAP-25 expression were a result of any change in neuritic outgrowth caused by the infection. It is therefore more likely that the increased protein expression reflects another type of reaction to the infection. Such effects of the infection could either be directly caused by the pathological isoform PrP^{Sc} or reflect a cellular response to the infection. In the former case, an alteration in the endocytotic machinery caused by PrP^{Sc}, which is present in lysosomes (32), could tentatively lead to dysfunctions in the endocytotic recycling pathway, and induce an increased expression of these proteins. On the other hand, an up-regulation of SNAP-25 protein expression has previously been observed in the brain as a response to kainate and colchicine exposure as well as axonal, mechanical lesions (33-37). The up-regulation of SNAP-25 was not related to sprouting or growth in some of these studies, since there was no paralelled increase in GAP-43 expression (34,36,37).

Furthermore, it has been shown that SNAP-25 plays an important role in dampening the calcium response evoked by depolarization with KCl in hippocampal glutamatergic neurons. Hippocampal GABAergic neurons have recently been demonstrated to lack SNAP-25 expression. Upon transfection with this protein, lower calcium responses were seen in these neurons when depolarized with KCl (38). Thus, SNAP-25 may play a neuroprotective role in preventing an exaggerated glutamate release that could be excitotoxic. Although the GT1-1 cells are not glutamatergic, but GABAergic, an up-regulation of SNAP-25 could still reflect a protective cell response to diminish neurotransmitter release also in these cells, since GABA functions as an excitatory transmittor in GT1-1 cells (39,40). Also *in vivo*, GABA is an excitatory transmittor in GnRH neurons (41). Decreased KCl-evoked N-type calcium channel responses has previously been described using fluorometric calcium measurements in the GT1-1 cell system after scrapie infection (12). The increased SNAP-25 expression in

ScGT1-1 cells might be implicated in the reduced KCl-evoked calcium responses, similar to the situation described in hippocampal neurons.

In addition, syntaxin 1A binding with the synaptic protein interaction (synprint) site in voltage-gated N-type calcium channels can modulate gating and reduce inward currents in expression systems (16,18,42,43) as well as in isolated nerve terminals (15). SNAP-25b interaction with the synprint site also reduces inward currents when co-expressed with N-type calcium channels in tsA-201 cells (18). The importance of a balance between synaptic vesicle release proteins has been suggested since the co-expression of SNAP-25b counteracts modulatory effects induced by syntaxin 1A (18). Therefore, disturbances in the balance of SNAP-25 and syntaxin 1A expression might be involved in the reduced N-type calcium responses described in ScGT1-1 cells. The increased expression of synaptophysin, which is localized to the membrane of small synaptic vesicles in neurons and neuroendocrine cells (44-47) is of interest, since this indicates that the synaptic vesicles containing classical neurotransmittors may be affected in scrapie-infected neurons. The function of synaptophysin is unclear and no essential changes in the neurotransmittor release could be observed in knockout mice (48). However, an interaction between synaptophysin and synaptobrevin has been demonstrated indicating implications in the control of exocytosis (49). This interaction has also been suggested to be important for synaptic vesicle maturation (50). In addition, inhibition of SNAP-25 expression using antisense has previously been shown to induce decreased expression of synaptophysin (29) indicating that the expression of these proteins may be related.

Prion diseases are associated with synaptic disorganization and reduction as seen in brains from both patients with CJD (3,4) and scrapie-infected mice (7,51-54). The degeneration and loss of axon terminals occur early during scrapie infections and in mice it precedes a dendritic spine loss and neuronal cell death (51,53,54). The loss of axon terminals

is paralelled by decreased levels of several presynaptic proteins in both CJD (5,6) and in murine scrapie (8). In the latter disease no marked cell death in the brain could be seen, but an increased level of c-jun/AP-1, mainly in reactive microglial cells, indicated phagocytosis of exogenous elements including synaptic debris (8).

In conclusion, marked changes were observed in the expression of SNAP-25, synaptophysin and syntaxin 1A in our cell system following scrapie infection. These clearly demonstrate that a scrapie infection can cause changes in the expression of proteins involved in neuronal secretion, which may be implicated in the reduced N-type calcium channel responses described previously in the scrapie-infected GT1-1 cells. In addition, these changes observed might be involved in the pathogenesis of synaptic alterations that are prominent features of prion diseases.

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Figure legends

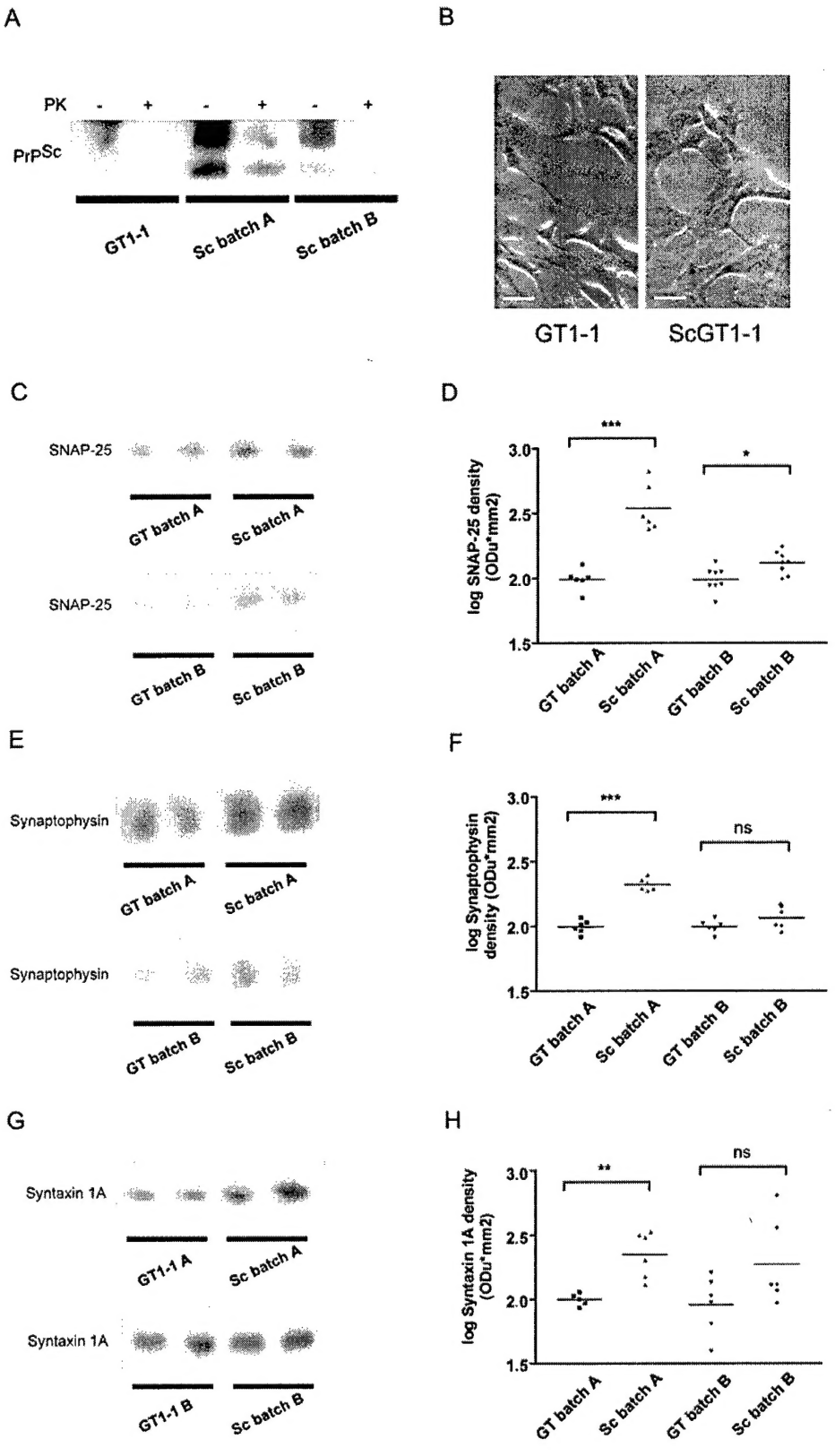
Fig. 1. The expression of neurosecretory proteins in GT1-1 cells infected with scrapie (RML) homogenate at two different time points (Sc batch A and B). (A) Western blot showing bands characteristic for PrP^{Sc} after proteinase K (PK) treatment. (B) No difference in morphology is seen between ScGT1-1 cells (Sc batch A) and uninfected GT1-1 cells. Phase contrast microscopy, bar 20μm. (C, E, G) Western blots showing SNAP-25, synaptophysin and syntaxin 1A expression in Sc batch A and B cells and corresponding uninfected GT1-1 cells. (D, F, H) Scatter diagrams showing the expression of SNAP-25, synaptophysin and syntaxin 1A distribution (logarithmic, optical density values) in Sc batch A cells, 60-85 days p.i; Sc batch B cells 65-70 days p.i., and the corresponding uninfected GT1-1 cells. A significantly increased expression of SNAP-25 (D), synaptophysin (F) and syntaxin 1A (H) was observed in the Sc batch A cells containing the higher level of PrP^{Sc}, while a more modest, but significant, increase in expression of SNAP-25 could be seen in the Sc batch B cells (D). No increased expression of synaptophysin or syntaxin 1A could be seen in the Sc batch B cells (F, H). (Bar = mean values; Student's *t*-test together with Welch's corrections *** $P<0.001$; ** $P<0.01$; * $P<0.05$).

Fig. 2. Relative mRNA levels of the SNAP-25a and b isoforms in ScGT1-1 cells (Sc batch A; 100 days p.i.) compared to mRNA levels in corresponding, uninfected GT1-1 cells using real-time PCR. The mRNA level of cyclophilin is used as an endogenous control. Both SNAP-25a and b isoform transcripts are significantly increased in ScGT1-1 cells. (Bar = mean values; Student's *t*-test *** $P<0.001$; ** $P<0.01$; * $P<0.05$).

Fig. 3. ScGT1-1 (Sc batch A; 90 days p.i.) and uninfected GT1-1 cells treated or not treated with 1 mM db-cAMP and 200 μM IBMX for 3 days and thereafter fixed and immunolabelled for SNAP-25 or synaptophysin antibodies. SNAP-25 is concentrated to the plasma membrane both in GT1-1 cells (A) and ScGT1-1 cells (B). The synaptophysin distribution shows a fine punctate pattern over the cell soma in uninfected GT1-1 cells (C) and in ScGT1-1 cells (D). Treatment with cAMP and IBMX induced neuritic outgrowth in both GT1-1 (E) and ScGT1-1 (F) cells and the SNAP-25 localization was similar in these cells. Bar 25μm.
Exposure to db-cAMP and IBMX induced an increased expression of SNAP-25 (G) and synaptophysin (H) in both GT1-1 and ScGT1-1 cells as determined by Western blotting.

Fig. 4 ScGT1-1 cells and uninfected GT1-1 cells treated with 0.5μM quinacrine for 2-3 weeks and analysed for PrP^{Sc} by Western blotting. (A) No bands, characteristic for PrP^{Sc}, are detected in the quinacrine-treated (+Q) ScGT1-1 cells (Sc batch A; 85 days p.i). (B) The expression of SNAP-25 and synaptophysin in the quinacrine-treated ScGT1-1 cells was lower than in non-treated (-Q) ScGT1-1 cells and comparable to that in treated or not treated uninfected GT1-1 cells.

Fig. 1


A
PK - + - + - +
PrPSc
GT1-1
Sc batch A
Sc batch B
B
GT1-1
ScGT1-1
C
SNAP-25
GT batch A
Sc batch A
SNAP-25
GT batch B
Sc batch B
D
log SNAP-25 density (ODu*mm2)
3.0
2.5
2.0
1.5
***
*
GT batch A
Sc batch A
GT batch B
Sc batch B
E
Synaptophysin
GT batch A
Sc batch A
Synaptophysin
GT batch B
Sc batch B
F
log Synaptophysin density (ODu*mm2)
3.0
2.5
2.0
1.5
***
ns
GT batch A
Sc batch A
GT batch B
Sc batch B
G
Syntaxin 1A
GT1-1 A
Sc batch A
Syntaxin 1A
GT1-1 B
Sc batch B
H
log Syntaxin 1A density (ODu*mm2)
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2.5
2.0
1.5
**
ns
GT batch A
Sc batch A
GT batch B
Sc batch B

Fig. 2

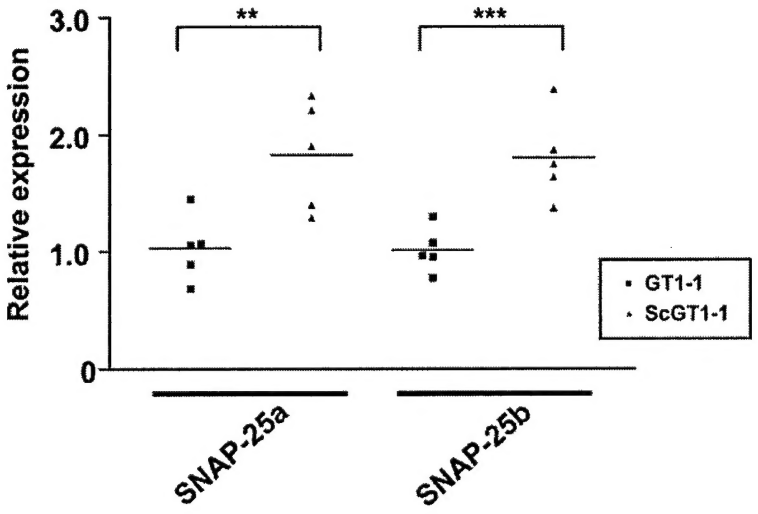
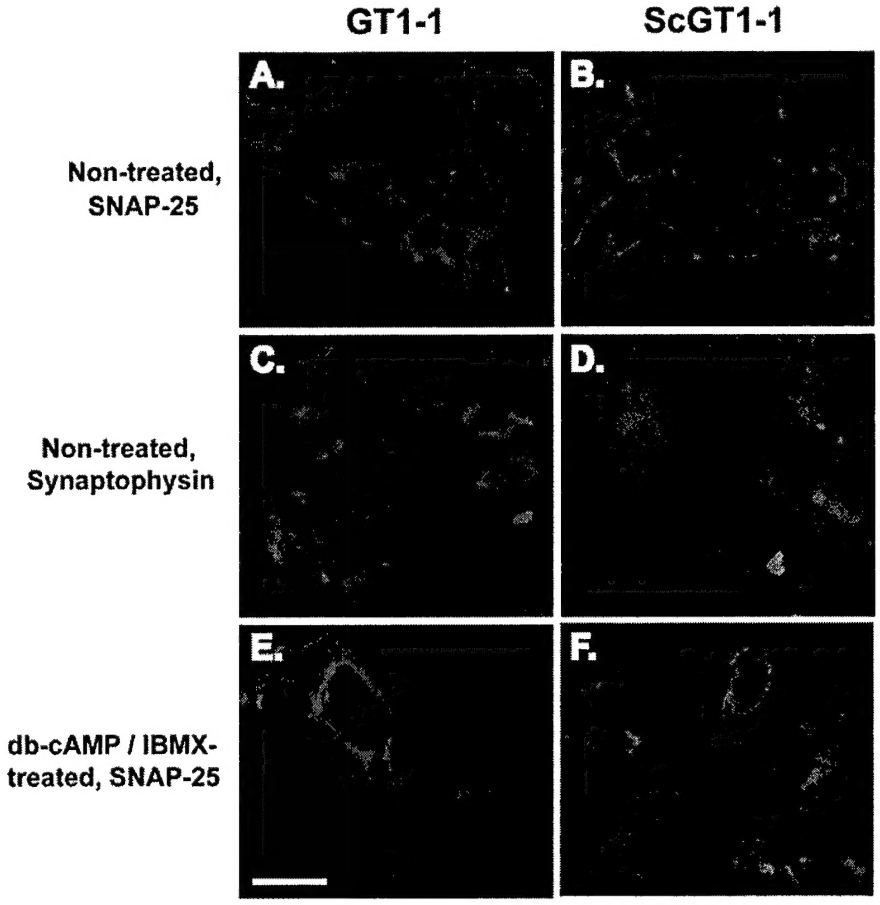
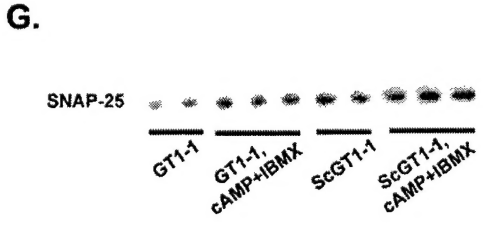


Fig. 3


GT1-1
ScGT1-1
A.
B.
Non-treated, SNAP-25
C.
D.
Non-treated, Synaptophysin
E.
F.
db-cAMP / IBMX-treated, SNAP-25


G.
SNAP-25
GT1-1
GT1-1, cAMP+IBMX
ScGT1-1
ScGT1-1, cAMP+IBMX

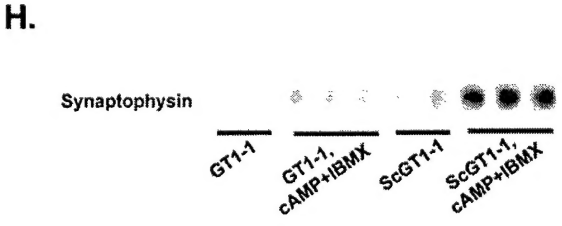
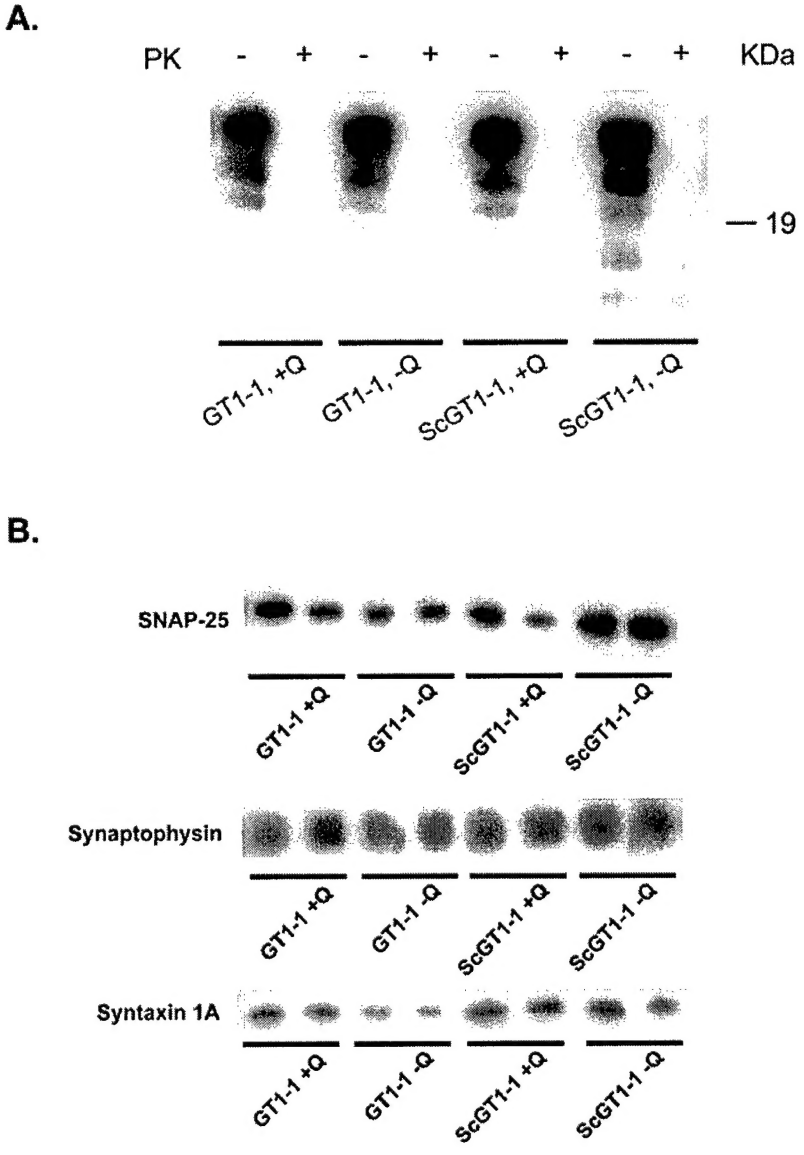

H.
Synaptophysin
GT1-1
GT1-1, cAMP+IBMX
ScGT1-1
ScGT1-1, cAMP+IBMX

Fig. 4


A.
PK - + - + - + - + KDa
19
GT1-1, +Q
GT1-1, -Q
ScGT1-1, +Q
ScGT1-1, -Q
B.
SNAP-25
Synaptophysin
Syntaxin 1A
GT1-1 +Q
GT1-1 -Q
ScGT1-1 +Q
ScGT1-1 -Q